UNIVERSITY OF BIRMINGHAM
University of Birmingham Research at Birmingham

Differential responses to vitamin D2 and vitamin d3 are associated with variations in free 25-hydroxyvitamin D

Chun, Rene; Hernandez, Ivan; Pereira, Renata; Swinkles, Leon; Huijs, Tonnie; Zhou, Rui; Liu, Nancy; Shieh, Albert; Guemes, Miriam; Mallya, Sanjay; Adams, John; Hewison, Martin

DOI:
10.1210/en.2016-1139

License:
None: All rights reserved

Document Version
Peer reviewed version

Citation for published version (Harvard):
Chun, R, Hernandez, I, Pereira, R, Swinkles, L, Huijs, T, Zhou, R, Liu, N, Shieh, A, Guemes, M, Mallya, S, Adams, J & Hewison, M 2016, 'Differential responses to vitamin D2 and vitamin d3 are associated with variations in free 25-hydroxyvitamin D', *Endocrinology*, vol. 157, no. 9, pp. 3420-3430. https://doi.org/10.1210/en.2016-1139

Link to publication on Research at Birmingham portal

Publisher Rights Statement:
Final Version of Record available at: http://dx.doi.org/10.1210/en.2016-1139

General rights
Unless a licence is specified above, all rights (including copyright and moral rights) in this document are retained by the authors and/or the copyright holders. The express permission of the copyright holder must be obtained for any use of this material other than for purposes permitted by law.

•Users may freely distribute the URL that is used to identify this publication.
•Users may download and/or print one copy of the publication from the University of Birmingham research portal for the purpose of private study or non-commercial research.
•User may use extracts from the document in line with the concept of 'fair dealing' under the Copyright, Designs and Patents Act 1988 (?)
•Users may not further distribute the material nor use it for the purposes of commercial gain.

Where a licence is displayed above, please note the terms and conditions of the licence govern your use of this document.

When citing, please reference the published version.

Take down policy
While the University of Birmingham exercises care and attention in making items available there are rare occasions when an item has been uploaded in error or has been deemed to be commercially or otherwise sensitive.

If you believe that this is the case for this document, please contact UBIRA@lists.bham.ac.uk providing details and we will remove access to the work immediately and investigate.

Download date: 04. May. 2023

Manuscript (MUST INCLUDE TITLE PAGE AND ABSTRACT) Click here to download Manuscript (MUST INCLUDE TITLE PAGE AND ABSTRACT) Chun et al final no figs.docx

Differential responses to vitamin D2 and vitamin D3 are associated with variations in free 25-hydroxyvitamin D

Rene F Chun[1], Ivan Hernandez[2], Renata Pereira[3], Leon Swinkles[4], Tonnie Huijs[4], Rui Zhou[1], Nancy Q Liu[1], Albert Shieh[1], Miriam Guemes[5], Sanjay M. Mallya[5], John S Adams[1], Martin Hewison[1,2,6].

[1]Dept of Orthopaedic Surgery, David Geffen School of Medicine at UCLA, Los Angeles, CA, 90095, USA
[2]Institute of Metabolism and Systems Research, The University of Birmingham, Birmingham B15 2TT, UK
[3]Dept of Pediatric Nephrology, David Geffen School of Medicine at UCLA, Los Angeles, CA, 90095, USA
[4]Future Diagnostics, Wijchen, 6603 BN, The Netherlands
[5]Section of Oral and Maxillofacial Radiology, UCLA School of Dentistry, Los Angeles, CA 90095, USA
[6]Centre for Endocrinology, Diabetes and Metabolism, Birmingham Health Partners, Birmingham, B15 2TT, UK

Abbreviated title: Vitamin D2 and D3 and free 25-hydroxyvitamin D
Key terms: Vitamin D2, vitamin D3, free vitamin D, vitamin D binding protein, bone, extra-skeletal, mouse
Word count: 4581
Tables: 2
Figures: 7

Address for correspondence:
Martin Hewison PhD
Institute of Metabolism & Systems Research
Level 2, IBR, Rm 225
The University of Birmingham
Birmingham, B15 2TT
UK

email: m.hewison@bham.ac.uk
Tel: 44 (0)121 414 6908 Fax: 44 (0) 121 415 8712

Disclosure statement: The authors have nothing to disclose

Abstract

25-hydroxyvitamin D (25D) circulates bound primarily to serum vitamin D binding protein (DBP), with DBP showing higher binding affinity for 25D3 than 25D2. We therefore hypothesized that vitamin D2 (D2) promotes higher serum levels of unbound 25D (free 25D), with different functional responses, relative to vitamin D3 (D3). Week 3 (wk3) C56BL/6 mice were placed on diets containing either D2 or D3 alone (both 1000 IU/kg). At wk8 and wk16 D2 mice had only 25D2 in circulation (26.6±1.9 and 33.3±4.4 ng/ml), and D3 mice had only 25D3 (28.3±2.0 and 31.7±2.1 ng/ml). At wk 8 (44.5±6.4 vs. 62.4±11.6 pg/ml, $p<0.05$), and wk16 (78.4±12.6 vs. 95.5±11.6) D2 mice had lower serum 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) relative to D3 mice. By contrast, measured free 25D was significantly higher in D2 mice at wk8 (16.8±0.65 vs. 8.4±0.63 pg/ml, $p<0.001$) and wk16 (17.4±0.43 vs. 8.4±0.44, $p<0.001$). Two-way ANOVA of bone histomorphometry showed that wk8 D2 mice had significantly higher osteoclast surface/bone surface, eroded surface/bone surface, and mineral apposition rate compared to D3 mice. Osteoblast surface/bone surface was higher in wk8 D2 females but not wk8 D2 males. At wk16, D2 mice had significantly higher bone volume/total volume and trabecular number compared to D3 mice. Differences in bone phenotype were observed despite D2 mice reaching similar serum 25D levels and lower 1,25D levels compared to D3 mice. These data indicate that 25D2 binds less well to DBP than 25D3, with resulting higher levels of free 25D promoting differential effects on bone in mice exposed to D2 alone.

Word count: 250 (250 max)

Introduction

In recent years there has been considerable debate concerning the role of vitamin D in human health. A key feature of this has been the definition of 'optimal' levels of vitamin D, and the potential impact of vitamin D-deficiency on human disease (1). At present the vitamin D 'status' of any given individual is determined by the total serum concentration of pro-hormone 25-hydroxyvitamin D (25D)(2). However, this parameter remains controversial with target levels for circulating 25D varying considerably depending on the population and clinical endpoint being studied(3,4). It is also unclear how total serum 25D levels relate to physiological or disease responses given that 25D is an inactive form of vitamin D that must be metabolized to active 1,25-dihydroxyvitamin D (1,25D) to initiate transcriptional responses via the nuclear vitamin D receptor (VDR). This may be particularly important for the many non-classical, extra-skeletal effects of 1,25D that have been reported in recent years (5-8), where conversion of 25D to 1,25D and subsequent VDR signaling may take place in a tissue-specific manner (9).

In serum, vitamin D metabolites are bound primarily to the serum vitamin D binding (DBP) (10,11). This association is central to the reabsorption of 25D in the proximal nephron and its subsequent metabolism to active 1,25D (1,25D) via the enzyme 1α-hydroxylase (CYP27B1) (12,13), but binding to DBP does not appear to underpin the entry of 25D into many other tissues. Instead, for most extra-renal tissues the general assumption is that vitamin D metabolites are biologically active when they are free of serum binding, even though this fraction may be very small (11,14). This is likely to be crucial to vitamin D physiology because of the widespread extra-renal expression of *CYP27B1* (15), where localized synthesis of 1,25D and associated VDR responses may be highly dependent on the tissue availability of 25D that is not bound to DBP (16). The so-called 'free-hormone hypothesis' (17,18) has questioned the validity of total serum 25D measurement as an accurate marker of vitamin D function (19), and revived interest in DBP as a pivotal component of vitamin D physiology.

Genome-wide association studies have underlined the link between *DBP* genotype, circulating levels of DBP and serum 25D status (20). However, studies of *Dbp* knockout mice showed that although these mice had almost undetectable serum levels of total 25D, the mice were phenotypically normal when raised on a normal vitamin D diet (21). A potential explanation for this is that despite lower circulating levels of total 25D, *Dbp*-/- mice have similar serum concentrations of free 25D relative to wild type mice. With this observation in mind, we developed an alternative strategy for comparing the relative importance of total vs free serum 25D based on the differential binding affinity of vitamin D metabolites for DBP. Previous studies have shown that relative to 25D3, 25D2 has a lower binding affinity for DBP (22-24). This appears to be a key factor in the decreased serum half-life of 25D2 compared to 25D3 (25), and the increasing reluctance to use vitamin D2 for supplementation studies (26). To further characterize the relative impact of these two forms of 25D on physiological responses *in vivo*, we utilized mouse models where animals were raised from weaning on diets containing exclusively vitamin D2 or vitamin D3.

Materials and Methods

Cell culture. Studies *in vitro* were carried out using the MG63 human osteoblast cell line (ATCC, Manassas, VA) cultured in DMEM medium with 10% fetal bovine serum and incubated at 37^{o}C. For dose response to 25D2/D3 studies, cells were incubated in 2% human serum (HS) or 10% bovine serum albumin (BSA) for 6 hrs. After treatment, media was removed and TRIzol was added to extract RNA, cDNA was generated by Super Script III Reverse Transcriptase with random primers according to suggested manufacturer procedures. RT-PCR was used to amplify cDNA and quantify genes of interest. Cycle threshold values were determined by instrument software for genes of interest and were subtracted by the cycle threshold values of 18S rRNA to yield ΔCt values. Data is expressed as $\Delta\Delta$Ct relative to vehicle treated cultures.

Mice*.* 3-week old male and female C57BL/6 mice were obtained from Jackson Laboratories (Sacramento, CA) and placed on the AIN-76A diet supplemented with either 1000 IU/kg vitamin D2 (D2) or vitamin D3 (D3) (Research Diets, Inc., New Brunswick, NJ), until euthanasia at wk8 or wk16 of age. Protocols were approved by the Office of Animal Research Oversight at University of California, Los Angeles (University of California, Los Angeles Chancellor's Animal Research Committee (ARC), 2013-113-01). For each time point, 8 male and 8 female mice were allocated to D2 and D3 mice, with sample size based on previous studies of vitamin D-sufficient and deficient mice (27). Each animal was an experimental unit, but mice were housed four animals per cage and entry into the study was staggered so that only eight mice were sacrificed on any given week. For serum vitamin D metabolites, bone histomorphometry and spleen immune cell and gene expression studies, samples were analyzed blind. The primary outcome of this approach was to assess total and free serum concentrations of 25D2 and 25D3, with secondary outcomes being changes in bone and spleen markers.

Mice were housed within the CHS B-Floor mouse only barrier Specific Pathogen-Free facility at UCLA that utilizes static micro-isolator and ventilated racks with beta chip and/or corn cob bedding material for mouse cages. All operations staff were dedicated to this facility only. Mice received D2- or D3-specific chow, and filtered tap water was provided via water bottles. Investigators had direct access to the mice, and all manipulations were carried out in a cage change station. Investigators provided fresh diet on a twice-weekly basis (Monday and Friday). Vivarium staff changed water and bedding weekly and, along with investigators, observed mice for general health and well-being. Automatic light cycles (12 light/12 dark) are installed in all animal holding rooms along with 24/7 room environment monitoring devices.

Analysis of mouse serum. Serum concentrations of $25D_2$, and $25D_3$ were analyzed by Liquid Chromatography-Tandem Mass Spectrometry methods at Heartland Assays (Ames, IA). Serum concentrations of 1,25D were assessed by Radioimmunoassay (Heartland Assays). Serum free 25D was quantified by immunoassay at Future Diagnostics (Wijchen, Netherlands). This assay has 77% cross-reactivity with 25D2. PTH 1-84 was measured by ELISA (Immutopics International, San Clemente, CA). Serum DBP was measured using a mouse DBP serum quantikine ELISA kit (R&D Systems, Minneapolis, MN 55413), according to manufacturer's instructions.

Gene expression assays. Mouse kidney and spleen RNA was isolated by Trizol extraction and cDNA synthesized with Super Script III Reverse Transcriptase according to manufacturer protocols (Invitrogen Thermo Fisher Scientific, Grand Island, NY) with random primers. qRT-PCR analysis was performed on an MX-3005P instrument (Agilent, Santa Clara, CA) utilizing TaqMan system reagents. Specific probe/primer sets were as follows (all Invitrogen Thermo Fisher Scientific, Grand Island, NY): Human osteocalcin (*BGLAP*, Hs01587814_g1), mouse 25-hydroxyvitamin D-1α-hydroxylase (*Cyp27b1*) (Mm01165918_g1); mouse vitamin D-24-hydroxylase (*Cyp24a1*) (Mm00487244_m1); mouse vitamin D receptor (*Vdr*) (Mm00437297_m1); *CD11b* (Mm00434455_m1); *CD14* (Mm00438094_g1); Receptor activator of nuclear factor kappa-B ligand (*RANKL*) (Mm00441906_m1). Eukaryotic 18S rRNA (probe/primer 4319413E) was used as an internal calibrator housekeeping gene. Reactions were carried out as described previously (27).

Bone histomorphometry. Static and dynamic histomorphometry was carried out on 8 and 16 week old mice. Mice were injected intra-peritoneal in the afternoon with (12 mg/ml) calcein (Sigma-Aldrich, St. Louis, MO) at 20 mg/kg bodyweight 7- and 2-days prior to euthanasia as recommended by the UCLA Bone Histomorphometry Core Laboratory. Right femurs were

collected from each animal, fixed in 70% ethanol, dehydrated with xylene, and embedded in methyl methacrylate. Longitudinal sections (5 μm) of femur were prepared on a Microm microtome (Richard-Allan Scientific, Kalamazoo, MI), and stained with toluidine blue. Static parameters of bone formation and resorption were measured in a defined area between 181 μm and 725 μm from the growth plate using an OsteoMeasure morphometry system (Osteometrics, Atlanta, GA). Dynamic histomorphometry, mineralizing surface per bone surface and mineral apposition rate were measured in unstained sections under ultraviolet light, using a B-2A set long pass filter consisting of an excitation filter ranging from 450 to 490 nm, a barrier filter at 515 nm, and a dichroic mirror at 500 nm. Bone formation rate (BFR) was a calculated parameter. The terminology and units used are those recommended by the Histomorphometry Nomenclature Committee of the American Society for Bone and Mineral Research (28).

***Micro computed tomography (μCT) analyses*.** Following euthanasia, left femurs were harvested, the attached soft tissues carefully removed, and the bones were fixed in 10% buffered formalin phosphate and then stored in 70% ethanol at 4°C. Micro–computed tomography scans (μCT; Skyscan 1172, Aartselaar, Belgium) were acquired at 55 kVp and 72 μA at a resolution of 12 μm. Volumetric analysis was performed using the Skyscan software. For trabecular bone analyses, 200 slices per femur were measured, covering a total of 2.4 mm from the proximal growth plate to the shaft distally. The analysis of the secondary spongiosa begins at 0.048 mm below the most distal point of the primary spongiosa, which was defined as directly distal to the most distal portion of the growth plate. Nomenclature for the bone morphology parameters is as described (29).

Statistical analysis. Data for serum vitamin D metabolites, serum PTH, as well as tissue gene expression data were analyzed by student's *t*-test on Excel (Microsoft, Redmond, WA). Bone histomorphometry parameters were analyzed by Two-way ANOVA on Prizm 5 (GraphPad, La

Jolla, CA). Tables of data were presented as mean values ± standard error of the mean (SEM) and error bars in graphs were standard deviation (SD) or SEM as indicated in figure legends.

Results

Differential potency of 25D2 and 25D3 in vitro

Consistent with previous studies of human monocytes (16), analysis of MG63 osteoblastic cells *in vitro* showed that these cells were more sensitive to 25D (25D2 or 25D3) when cultured in the medium lacking serum (2% human serum, 2% HS) (**Figure 1**). Furthermore, MG63 cells cultured in 2% HS showed greater sensitivity to 25D2 relative to 25D3 when measuring induction of the mRNA for osteocalcin (OC) (**Figure 1A**). However, both 25D2 and 25D3 were equally effective in stimulating OC mRNA when MG63 cells were cultured in serum-free medium (**Figure 1B**). These data supported the over-arching hypothesis that 25D2 has greater biological potency in the presence of serum proteins such as DBP.

Serum free 25D is elevated in mice raised on vitamin D2 diet relative to vitamin D3

To assess possible variations in functionality of 25D2 and 25D3 *in vivo*, further studies were carried out using mice raised on diets containing either vitamin D2 (D2) or vitamin D3 (D3) only. These diets had no detrimental effect on mice at 8 or 16 wks of age, and each group of animals exhibited circulating 25D that was exclusively 25D2 or 25D3, with corresponding serum 1,25D2 or 1,25D3. In D2 mice, serum concentrations of 25D2 (26.56 ± 1.88 ng/ml at 8 wks and 33.25 ± 4.38 ng/ml at 16 wks) were similar to serum concentrations of 25D3 in D3 mice (28.27 ± 2.01 ng/ml at 8 wks and 31.72 ± 2.06 ng/ml at 16 wks) (**Figure 2A**). For both D2 and D3 mice, serum concentrations of 25D2 or 25D3 were significantly higher at 16 wks of age compared to 8 wks. In a similar fashion, serum concentrations of 1,25D2 and 1,25D3 were higher in wk 16 mice compared to wk 8 mice. However, in both age groups serum concentrations of 1,25D3 in D3 mice (62.38 ± 11.59 pg/ml at 8 wks and 95.53 ± 11.59 pg/ml at 16 wks), were significantly

higher than serum 1,25D2 in D2 mice (44.49 ± 6.45 pg/ml at 8 wks and 78.42 ± 12.61 pg/ml at 16 wks) (**Figure 2B**). Measurement of DBP concentrations for week 8 serum samples showed no significant difference between D2 and D3 mice (**Figure 2C**). Despite this, free 25D levels were higher in D2 mice relative to D3 mice (16.75 ± 0.65 pg/ml vs 8.40 ± 0.63 pg/ml at 8 wks and 17.38 ± 0.43 ng/ml vs 8.43 ± 0.44 pg/ml at 16 wks) (**Figure 2C**).

Differences in kidney gene expression in D2 versus D3 mice

Analysis of kidney tissue showed no significant effect of D2 or D3 diets on expression of mRNA for the vitamin D receptor (*Vdr*), 25-hydroxyvitamin D-1α-hydroxylase (*Cyp27b1*), or vitamin D-24-hydroxylase (*Cyp24a1*) in male or female mice at either wk8 or wk16 of age (**Figure 3**). Consistent with this, mean ± SD serum concentrations of PTH for D2 (193.16 ± 10.04 pg/ml females and 139.95 ± 7.24 pg/ml males) and D3 (195.69 ± 6.76 pg/ml females and 157.00 ± 5.32 pg/ml males) in wk8 mice showed no significant differences. Both male and female mice showed higher expression of *Vdr* (lower ΔCt) at wk16 of age compared to wk8 (**Figure 3A**). At 8 wks of age, D2 and D3 male mice showed lower expression of *Cyp27b1* (higher ΔCt) relative to their female counterparts (**Figure 3B**), and wk16 female D3 mice showed higher expression (lower ΔCt) of *Cyp24a1* relative to wk8 female D3 mice (**Figure 3C**).

Differences in spleen gene expression and immune cell composition in D2 versus D3 mice

To determine possible differential effects of D2 and D3 diets on extra-renal actions of 25D, analysis of mRNA expression was also carried out for spleen tissue. Data in **Figure 4A** indicate that male mice showed no significant change in expression for the genes *Cyp27b1, CD11b, CD14 and RANKL*. However, wk8 female D2 mice showed significantly increased expression of *Cyp27b1, CD11b, and RANKL*, and significantly lower expression of *CD14*. Further analysis of spleen tissue from wk8 female mice by flow cytometry showed that differential effects of D2 and

D3 were restricted to less abundant splenic cell populations such as natural killer (NK) cells and monocyte/macrophages (**Figure 4B**). In spleens from D2 females the percentage of NK cells (3.45 ± 0.36 vs 5.25 ± 0.31 % of $CD45^+$ cells) was lower, and the percentage of monocytes/macrophages was higher (3.31 ± 0.28 vs 2.40 ± 0.13 % of $CD45^+$ cells) relative to D3 females. Further analysis of the spleen monocyte population using CD11b/F4/80 markers showed a significantly higher proportion of CD11b-negative monocytes (10.62 ± 3.85 vs 3.10 ± 1.24) in D3 mice relative to D2 mice, but CD11b-positive monocytes were unaffected by diet.

Variations in bone structure and cell composition in D2 versus D3 mice

Histomorphometric analysis of femurs from D2 and D3 mice showed changes in bone structure that were sex, diet, and time-related. Example histomorphometric analyses for femur bone from wk16 female mice on D2 or D3 diets are shown in **Figure 5.** Quantitative analysis of bone structure markers was carried out for femur sections from multiple mice using defined regions of interest (**Figure 5** inset box) with resulting data shown in **Table 1**. Statistically significant differences in bone structure and turnover markers for wk8 mice were primarily due to sex, with male mice showing higher bone volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), mineralized bone surface (MS/BS), osteoid surface (OS/BS), mineral apposition rate (MAR), and bone formation rate (BFR/BS), while trabecular spacing (Tb.Sp) was lower in males relative to females. Interestingly, at this time point, 2-way ANOVA showed that the eroded bone surface (ES/BS) and MAR were significantly affected by D2 vs D3 diet. At wk16, all bone structure and turnover parameters were statistically different in male vs females irrespective of diet. However, there was a significant diet effect in that BV/TV, Tb.N, and Tb.Th were significantly higher in D2 vs D3 mice, with Tb.Sp being lower in D2 mice. Further analysis of bone structure by µCT in wk16 mice confirmed the differential effects of D2 and D3 on BV/TV, Tb.N, and Tp.sp with these effects predominating in male mice (**Figure 6**)**.**

Histomorphometric analyses for bone bone-resorbing osteoclasts (Oc) and bone-forming osteoblasts (Ob) was also carried out, as illustrated by the example femurs from wk8 female mice in shown in **Figure 7**. Quantitative analysis of these data using femur sections from wk8 and wk16 mice is shown in **Table 2**. In wk8 mice the primary factor influencing these markers was diet, with osteoclast number (N.Oc), osteoclasts per bone perimeter (N.Oc/B.Pm), number of osteoclasts per trabecular area (N.Oc/T.Ar), osteoclast surface per bone surface (Oc.S/BS), number of osteoblasts (N.Ob), number of osteoblasts per bone perimeter (N.Ob/B.Pm), number of osteoblasts per trabecular area (N.Ob/T.Ar), osteoblast surface per bone surface (Ob.S/BS) being significantly affected by D2 vs D3 diet. In wk8 females all of the osteoclast and osteoblast markers were higher in D2 mice relative to D3. In wk8 males osteoclast markers were also higher in D2 mice but there was no significant effect on osteoblast markers in the D2 mice (Mann-Whitney P=0.33). At wk16 D2 vs D3 diet had no significant effect on osteoclast and osteoblast markers but, in common with wk8 mice, sex of the animal was associated with variations in some makers, with N.Oc/B.Pm, Oc.S/BS, N.Ob, N.Ob/B.Pm, N.Ob/T.Ar, Ob.S/BS being higher in males vs females.

Discussion

The aim of the current study was to further analyze the impact of DBP on vitamin D bioavailability and physiology by generating an *in vivo* model where concentrations of free serum 25D were uncoupled from serum concentrations of total 25D. Previous studies have shown that *Dbp* knockout mice exhibit very low serum 25D and 1,25D due to elevated renal clearance of 25D and decreased conversion to 1,25D in proximal tubules (21). Despite this, they do not have an abnormal skeletal phenotype unless raised on a vitamin D-deficient diet (21). This has been attributed to the fact that although *Dbp* KO mice have low circulating levels of 25D and 1,25D, the bioactivity of these metabolites remains normal (30). Previous *in vitro* data from our group have shown that DBP acts to attenuate cellular responses to vitamin D

metabolites, with monocytes cultured in serum from *Dbp* knockout mice showing more sensitive responses to 25D and 1,25D (16). Other studies have reported increased sensitivity to 1,25D in osteoblastic cells cultured in medium containing serum from *Dbp* knockout mice (30). Here we showed enhanced sensitivity to 25D2 relative to 25D3 in human osteoblastic MG63 cells, but only in the presence of serum containing DBP. These data supported the central hypothesis of the current study that 25D2 binds to DBP with lower affinity than 25D3, and therefore exhibits greater bioavailability in the presence of DBP-containing serum. This was further endorsed by analysis of serum free 25D concentrations in D2 and D3 mice, where free 25D was significantly higher in D2 vs D3 mice, despite both groups of animals having similar concentrations of total 25D and DBP at each time point.

Both the D2 and D3 forms of vitamin D are commonly used in human supplementation studies, but the relative efficacy of the two isoforms continues to be the subject of widespread debate. A recent systematic review and meta-analysis indicated that vitamin D3 was more effective in raising serum concentrations of 25D relative to vitamin D2 (31). Other studies have reported similar enhancement of serum 25D levels with vitamin D2 or vitamin D3 supplementation, but better maintenance of the resulting elevated serum 25D was achieved with vitamin D3 (32). A potential explanation for data from both of these studies is that the lower binding affinity of DBP for 25D2 relative to 25D3 (22-24) may impair availability of the 25D2-DBP complex for megalin-mediated glomerular reabsorption (12). This was not supported by data in the current study, where there was no significant difference in serum total 25D concentrations for D2 versus D3 mice at wk8 or wk16. However, there was a trend towards higher serum concentrations of 1,25D in D3 mice (although this was only statistically significant in wk8 male mice), suggesting a greater conversion rate of 25D3 to 1,25D3, relative to the rate of conversion of 25D2 to 1,25D2. Previous studies have shown that there is no preferential metabolism of 25D2 to 1,25D2 vs

25D3 to 1,25D3 (33) although, in contrast to the current study, this particular analysis involved human subjects with a combination of D2 and D3 circulating metabolites.

The elevation of serum 1,25D in wk16 D3 mice relative to D2 mice may occur through better recovery of substrate for the proximal tubule 1α-hydroxylase enzyme via megalin-mediated glomerular reabsorption of the 25D3-DBP complex (12). The difference does not appear to be due to differential kidney expression of the enzyme 1α-hydroxylase, as analysis of renal mRNA for *Cyp27b1* showed no difference between D2 and D3 mice. In a similar fashion, expression of renal mRNA for *Cyp24a1* was not significantly different between D2 and D3 mice, suggesting that 25D2 and 25D3 were not subject to different levels of catabolism. Interestingly, in both D2 and D3 mice, serum concentrations of 1,25D were significantly higher in wk16 versus wk8 mice. The mechanism for this is unclear and does not appear to be due to any age-related changes in renal *Cyp27b1* and/or *Cyp24a1* expression.

The relative impact of 25D2 and 25D3 on serum concentrations of free 25D has been studied previously in human subjects supplemented with oral vitamin D2 or vitamin D3 at 1,000 IU/day for 3 months (34). In this instance, the subjects receiving vitamin D3 showed higher circulating levels of 25D and 1,25D at the end of the study, consistent with the data presented here for D3 versus D2 mice. In contrast to mouse data in the current study, human subjects supplemented with vitamin D2 showed lower levels of free or bioavailable (non-DBP bound) 25D relative to those supplemented with vitamin D3, although this effect was lost when data were adjusted for supplementation compliance (34). Other than species, the major difference between these two studies is that the mice described here received exclusively either D2 or D3 from weaning, and consequently their circulating 25D was made up entirely of either 25D2 or 25D3. In humans supplemented with D2 a significant proportion of 25D is made up of endogenously synthesized 25D3 obtained from cutaneous synthesis of vitamin D3 and vitamin D3 from food fortification. It

was also notable that the human cohort study outlined above (34) involved elderly hip fracture patients, who were assessed only for changes in serum calcium relative to free 25D. We did not measure serum calcium in the D2 or D3 mice. However, there was no significant difference in serum PTH levels between these mice suggesting that differences in free 25D (at least at the levels found in these mice) is not a significant component of vitamin D-mediated regulation of parathyroid function and calcium homeostasis.

Although male and female D2 mice showed similar levels of total serum free 25D irrespective of age, the differential effects of 25D2 versus 25D3 were age and sex-dependent. The selection of week 8 and week 16 ages for assessment of bone phenotype was intended to capture data from the C57BL/6 mice at different stages of bone development: 1) at a young adult stage (week 8) when BV/TV is toward its peak; 2) at a mature adult state (week 16) when BV/TV begins to decline (35). At wk8, 25D2 was associated with enhanced osteoclastic and osteoblastic activity, but this effect was most pronounced in female mice. Temporal variations in bone development for male and female mice have been reported previously (35), and these observations may explain the differential effects of D2 and D3 demonstrated in the current study. Specifically, it is possible that osteoclast/osteoblast effects of free 25D are strongest in younger mice during active skeletal development, with these effects being lost later in life. This would also explain the stronger impact of D2 versus D3 in female mice, where changes in parameters of skeletal development tend to be slower than in male counterparts. In this setting greater bioavailability of 25D2 may promote stronger effects in females relative to 25D3 because of generally higher bone cell activity at females at this age point. As bone remodeling is the net effect of the activities of bone forming and bone resorbing cells, differences in bone structure will take longer to develop, and this may explain the significant differences in markers such as BV/TV, Tb.N and Tb. Sp for D2 versus D3 at week 16, but not at week 8.

Variable responses to 25D2 and 25D3 may not be restricted to the skeleton, as female D2 mice also exhibited differential effects on spleen gene expression and immune cell composition. Previous reports have shown that the spleen serves as a reservoir for monocyte/macrophage-lineage osteoclast precursors (OCP), which are generated and transferred to bone in response to diverse stimuli including vitamin D (36). Data presented here showed that D2 diet was associated with higher mRNA expression levels of monocyte/macrophage specific markers *CD11b* and *RANKL*, and also changes in the total monocyte population within spleen. Although further experiments must be completed in order to determine the specific cell subpopulations affected by D2 treatment, the results suggest a link between altered osteoblast/osteoclast markers in vitamin D2 vs D3 mice and the increase in OCP markers in the spleen. Therefore, the spleen may be a target tissue for the enhanced levels of free 25D observed in D2 mice. Further *in vivo* studies using stimulated murine models will help elucidate whether these changes are more pronounced following immune challenge.

Studies in human subjects have reported better correlation with serum free 25D relative to total serum 25D for bone mineral density in healthy subjects (37), and serum markers of mineral homeostasis in renal dialysis patients (38). However, these data have not been consistent and recent data for bone density and mineral metabolism markers in healthy adults suggested that analysis of free 25D does not significantly improve correlations relative to conventional measurement of total serum (39). This may be due, in part, to existing strategies for calculating free or bioavailable 25D based on measurement of serum DBP levels, where discrepancies between ELISA analyses using monoclonal versus polyclonal antibodies have been described, particularly in the context of racial variations in DBP phenotype (40). The advent of new assays for direct measurement of free 25D (41), as documented in the current study, has enabled a clearer appraisal of the functional impact of this form of vitamin D. To date, measurement or estimation of free serum 25D concentrations has been primarily focused on parameters

associated with classical skeletal actions of vitamin D. Although the data presented here support a role for free 25D in defining some parameters of skeletal function, it seems likely that extra-skeletal tissues will also be important targets for free 25D. Previous studies *in vitro* (16,42) and spleen analyses reported here suggest that cells from the immune system may be strongly influenced by 25D that is not bound to DBP. As such, it will be interesting in future studies to determine whether, relative to vitamin D3, vitamin D2 supports enhanced immunomodulatory actions, either in the form of enhanced antibacterial or anti-inflammatory actions. Finally, it will be important in future studies to determine the extent to which variations in DBP binding affinity influence vitamin D metabolites other than 25D. This includes 1,25D, where variations in synthesis of the active form of vitamin D may play a key role in mediating both skeletal and extra-skeletal responses.

Acknowledgements

Authors wish to thank the following for technical assistance in different phases of this project: Emaad Farooqui and Niklas Eriksson in MG63 qPCR experiments and Timothy Lee and Kereat Grewal in qPCR experiments of mouse kidney. This work is supported in part by a research grant from Investigator-Initiated Studies Program of Merck Sharp & Dohme Corp (project number IISP 51039). The opinions expressed in this paper are those of the authors and do not necessarily represent those of Merck Sharp & Dohme Corp.

Legends to figures

Figure 1. Effects of $25D_2$ or $25D_3$ on osteocalcin mRNA in osteoblastic cells. MG63 cells were treated with $25D_2$ (o) or D_3 (●) (0-200 nM) in medium containing: A. 2% human serum (HS); B. 10% BSA for 6 hrs. Data show increased mRNA for the osteoblastic marker gene osteocalcin (OCN). Values are mean ± SD, n= 4 cell preparations. *** = P<0.001.

Figure 2. Vitamin D metabolites in mice raised on diets containing vitamin D2 or vitamin D3 only. Serum concentrations of: A) total 25-hydroxyvitamin D (25D) (either 25D2 or 25D3); B) 1,25-dihydroxyvitamin D (1,25D) (either 1,25D2 or 1,25D3); C) serum vitamin D binding protein (DBP); D) free 25D in male or female mice raised on vitamin D2 (D2)- or vitamin D3 (D3)-only diets from wk 3 of age until either 8 wks or 16 wks of age. N = 3 for each male/female group. * = statistically different from equivalent 8 wk old mice, p<0.05, *** = statistically different from equivalent 8 wk old mice, p<0.001. ### = statistically different from D3 equivalent, p<0.001. n.d. = not done.

Figure 3. Effect of vitamin D2 or vitamin D3 on kidney gene expression in mice. Expression of mRNA for the vitamin D receptor (*Vdr*), 25-hydroxyvitamin D-1α-hydroxylase (*Cyp27b1*), and 24-hydroxylase (*Cyp24a1*) in kidneys from male or female mice raised on vitamin D2 (D2)- or vitamin D3 (D3)-only diets from wk 3 of age until either 8 wks or 16 wks of age. N = 6 separate animals. * = statistically different from equivalent 8 wk old mice, p<0.05, *** = statistically different from equivalent 8 wk old mice, p<0.001. ΔΔ = statistically different from equivalent female mice, p<0.01.

Figure 4. Effect of vitamin D2 or vitamin D3 on spleen gene expression and immune cell composition in week 8 female mice. Female mice raised on vitamin D2 (D2)- or vitamin D3 (D3)-only diets from wk 3 of age until either 8 wks or 16 wks of age were assessed for: A.

Expression of mRNA for 25-hydroxyvitamin D-1α-hydroxylase (*Cyp27b1*), *CD11b*, CD14, and RANK ligand (*RANKL*). Values are shown as fold-change in mRNA for each gene in D2 mice relative to D3 mice (D3 mice = 1); B. Immune cell composition based on % of cells expressing the immune cell surface antigens CD4 (helper T cells), CD8 (cytotoxic T cells), B220 (B cells), NK1.1 (natural killer cells), and F4/80 and CD11b (monocytes). N = 6 separate animals. * = statistically different from equivalent D3 mice, $p<0.05$, *** = statistically different from D3 mice, $p<0.001$.

Figure 5. Bone structure in female mice on vitamin D2 and vitamin D diets at week 16 of age. Example images from bone histomorphometric analysis (x40 magnification) of toluidine blue stained femurs from wk16 female mice fed vitamin D3 (D2) or vitamin D3 (D3) diets. Black box indicates region of interest for quantitative analysis.

Figure 6. Analysis of bone structure by µCT. A) Example µCT scans for wk 16 male mice on vitamin D2 (D2) or vitamin D3 (D3) diets. B) Quantification of bone volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), and trabecular spacing (Tb.Sp) in femurs from wk16 male and female mice. Data are mean (bar) of n=3 left femurs. * = statistically different (*t*-test) from D3 mice, $p < 0.05$, ** $p < 0.01$.

Figure 7. Osteoclast and osteoblast markers in female mice on vitamin D2 and vitamin D diets at week 8 of age. Example images from bone histomorphometric analysis (x 200 magnification) of toluidine blue stained femurs from wk8 female mice fed vitamin D2 (D2) and vitamin D3 (D3) diets.

References

1. Holick MF. Vitamin D deficiency. N Engl J Med 2007; 357:266-281

2. Holick MF. Vitamin D status: measurement, interpretation, and clinical application. Ann Epidemiol 2009; 19:73-78

3. Ross AC, Manson JE, Abrams SA, Aloia JF, Brannon PM, Clinton SK, Durazo-Arvizu RA, Gallagher JC, Gallo RL, Jones G, Kovacs CS, Mayne ST, Rosen CJ, Shapses SA. The 2011 report on dietary reference intakes for calcium and vitamin D from the Institute of Medicine: what clinicians need to know. The Journal of clinical endocrinology and metabolism 2011; 96:53-58

4. Holick MF, Binkley NC, Bischoff-Ferrari HA, Gordon CM, Hanley DA, Heaney RP, Murad MH, Weaver CM. Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline. The Journal of clinical endocrinology and metabolism 2011; 96:1911-1930

5. Adams JS, Hewison M. Unexpected actions of vitamin D: new perspectives on the regulation of innate and adaptive immunity. Nat Clin Pract Endocrinol Metab 2008; 4:80-90

6. Carlberg C, Seuter S. A genomic perspective on vitamin D signaling. Anticancer research 2009; 29:3485-3493

7. Spina CS, Tangpricha V, Uskokovic M, Adorinic L, Maehr H, Holick MF. Vitamin D and cancer. Anticancer research 2006; 26:2515-2524

8. Holick MF. Vitamin D: importance in the prevention of cancers, type 1 diabetes, heart disease, and osteoporosis. Am J Clin Nutr 2004; 79:362-371

9. Hewison M. Vitamin D and immune function: autocrine, paracrine or endocrine? Scand J Clin Lab Invest Suppl 2012; 243:92-102

10. Bikle DD, Gee E, Halloran B, Kowalski MA, Ryzen E, Haddad JG. Assessment of the free fraction of 25-hydroxyvitamin D in serum and its regulation by albumin and the vitamin D-binding protein. The Journal of clinical endocrinology and metabolism 1986; 63:954-959

11. Bikle DD, Gee E, Halloran B, Haddad JG. Free 1,25-dihydroxyvitamin D levels in serum from normal subjects, pregnant subjects, and subjects with liver disease. The Journal of clinical investigation 1984; 74:1966-1971

12. Nykjaer A, Dragun D, Walther D, Vorum H, Jacobsen C, Herz J, Melsen F, Christensen EI, Willnow TE. An endocytic pathway essential for renal uptake and activation of the steroid 25-(OH) vitamin D3. Cell 1999; 96:507-515

13. Nykjaer A, Fyfe JC, Kozyraki R, Leheste JR, Jacobsen C, Nielsen MS, Verroust PJ, Aminoff M, de la Chapelle A, Moestrup SK, Ray R, Gliemann J, Willnow TE, Christensen EI. Cubilin dysfunction causes abnormal metabolism of the steroid hormone 25(OH) vitamin D(3). Proceedings of the National Academy of Sciences of the United States of America 2001; 98:13895-13900

14. Bouillon R, Van Assche FA, Van Baelen H, Heyns W, De Moor P. Influence of the vitamin D-binding protein on the serum concentration of 1,25-dihydroxyvitamin D3. Significance of the free 1,25-dihydroxyvitamin D3 concentration. The Journal of clinical investigation 1981; 67:589-596

15. Zehnder D, Bland R, Williams MC, McNinch RW, Howie AJ, Stewart PM, Hewison M. Extrarenal expression of 25-hydroxyvitamin d(3)-1 alpha-hydroxylase. The Journal of clinical endocrinology and metabolism 2001; 86:888-894

16. Chun RF, Lauridsen AL, Suon L, Zella LA, Pike JW, Modlin RL, Martineau AR, Wilkinson RJ, Adams J, Hewison M. Vitamin D-binding protein directs monocyte responses to 25-hydroxy- and 1,25-dihydroxyvitamin D. The Journal of clinical endocrinology and metabolism 2010; 95:3368-3376

17. Mendel CM. The free hormone hypothesis: a physiologically based mathematical model. Endocr Rev 1989; 10:232-274
18. Hammond GL. Access of reproductive steroids to target tissues. Obstet Gynecol Clin North Am 2002; 29:411-423
19. Chun RF, Peercy BE, Orwoll ES, Nielson CM, Adams JS, Hewison M. Vitamin D and DBP: the free hormone hypothesis revisited. The Journal of steroid biochemistry and molecular biology 2014; 144 Pt A:132-137
20. Wang TJ, Zhang F, Richards JB, Kestenbaum B, van Meurs JB, Berry D, Kiel DP, Streeten EA, Ohlsson C, Koller DL, Peltonen L, Cooper JD, O'Reilly PF, Houston DK, Glazer NL, Vandenput L, Peacock M, Shi J, Rivadeneira F, McCarthy MI, Anneli P, de Boer IH, Mangino M, Kato B, Smyth DJ, Booth SL, Jacques PF, Burke GL, Goodarzi M, Cheung CL, Wolf M, Rice K, Goltzman D, Hidiroglou N, Ladouceur M, Wareham NJ, Hocking LJ, Hart D, Arden NK, Cooper C, Malik S, Fraser WD, Hartikainen AL, Zhai G, Macdonald HM, Forouhi NG, Loos RJ, Reid DM, Hakim A, Dennison E, Liu Y, Power C, Stevens HE, Jaana L, Vasan RS, Soranzo N, Bojunga J, Psaty BM, Lorentzon M, Foroud T, Harris TB, Hofman A, Jansson JO, Cauley JA, Uitterlinden AG, Gibson Q, Jarvelin MR, Karasik D, Siscovick DS, Econs MJ, Kritchevsky SB, Florez JC, Todd JA, Dupuis J, Hypponen E, Spector TD. Common genetic determinants of vitamin D insufficiency: a genome-wide association study. Lancet 2010; 376:180-188
21. Safadi FF, Thornton P, Magiera H, Hollis BW, Gentile M, Haddad JG, Liebhaber SA, Cooke NE. Osteopathy and resistance to vitamin D toxicity in mice null for vitamin D binding protein. The Journal of clinical investigation 1999; 103:239-251
22. Belsey R, Clark MB, Bernat M, Glowacki J, Holick MF, DeLuca HF, Potts JT, Jr. The physiologic significance of plasma transport of vitamin D and metabolites. Am J Med 1974; 57:50-56
23. Jones G, Byrnes B, Palma F, Segev D, Mazur Y. Displacement potency of vitamin D2 analogs in competitive protein-binding assays for 25-hydroxyvitamin D3, 24,25-dihydroxyvitamin D3, and 1,25-dihydroxyvitamin D3. The Journal of clinical endocrinology and metabolism 1980; 50:773-775
24. Hollis BW. Comparison of equilibrium and disequilibrium assay conditions for ergocalciferol, cholecalciferol and their major metabolites. J Steroid Biochem 1984; 21:81-86
25. Jones KS, Assar S, Harnpanich D, Bouillon R, Lambrechts D, Prentice A, Schoenmakers I. 25(OH)D2 half-life is shorter than 25(OH)D3 half-life and is influenced by DBP concentration and genotype. The Journal of clinical endocrinology and metabolism 2014; 99:3373-3381
26. Houghton LA, Vieth R. The case against ergocalciferol (vitamin D2) as a vitamin supplement. Am J Clin Nutr 2006; 84:694-697
27. Liu NQ, Ouyang Y, Bulut Y, Lagishetty V, Chan SY, Hollis BW, Wagner C, Equils O, Hewison M. Dietary vitamin D restriction in pregnant female mice is associated with maternal hypertension and altered placental and fetal development. Endocrinology 2013; 154:2270-2280
28. Parfitt AM, Drezner MK, Glorieux FH, Kanis JA, Malluche H, Meunier PJ, Ott SM, Recker RR. Bone histomorphometry: standardization of nomenclature, symbols, and units. Report of the ASBMR Histomorphometry Nomenclature Committee. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research 1987; 2:595-610
29. Bouxsein ML, Boyd SK, Christiansen BA, Guldberg RE, Jepsen KJ, Muller R. Guidelines for assessment of bone microstructure in rodents using micro-computed tomography. Journal of bone and mineral research 2010; 25:1468-1486

30. Zella LA, Shevde NK, Hollis BW, Cooke NE, Pike JW. Vitamin D-binding protein influences total circulating levels of 1,25-dihydroxyvitamin D3 but does not directly modulate the bioactive levels of the hormone in vivo. Endocrinology 2008; 149:3656-3667

31. Tripkovic L, Lambert H, Hart K, Smith CP, Bucca G, Penson S, Chope G, Hypponen E, Berry J, Vieth R, Lanham-New S. Comparison of vitamin D2 and vitamin D3 supplementation in raising serum 25-hydroxyvitamin D status: a systematic review and meta-analysis. Am J Clin Nutr 2012; 95:1357-1364

32. Oliveri B, Mastaglia SR, Brito GM, Seijo M, Keller GA, Somoza J, Diez RA, Di Girolamo G. Vitamin D3 seems more appropriate than D2 to sustain adequate levels of 25OHD: a pharmacokinetic approach. European journal of clinical nutrition 2015; 69:697-702

33. Hartwell D, Hassager C, Christiansen C. Effect of vitamin D2 and vitamin D3 on the serum concentrations of 1,25(OH)2D2, and 1,25(OH)2D3 in normal subjects. Acta endocrinologica 1987; 115:378-384

34. Glendenning P, Chew GT, Inderjeeth CA, Taranto M, Fraser WD. Calculated free and bioavailable vitamin D metabolite concentrations in vitamin D-deficient hip fracture patients after supplementation with cholecalciferol and ergocalciferol. Bone 2013; 56:271-275

35. Glatt V, Canalis E, Stadmeyer L, Bouxsein ML. Age-related changes in trabecular architecture differ in female and male C57BL/6J mice. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research 2007; 22:1197-1207

36. Nakamichi Y, Mizoguchi T, Arai A, Kobayashi Y, Sato M, Penninger JM, Yasuda H, Kato S, DeLuca HF, Suda T, Udagawa N, Takahashi N. Spleen serves as a reservoir of osteoclast precursors through vitamin D-induced IL-34 expression in osteopetrotic op/op mice. Proceedings of the National Academy of Sciences of the United States of America 2012; 109:10006-10011

37. Powe CE, Ricciardi C, Berg AH, Erdenesanaa D, Collerone G, Ankers E, Wenger J, Karumanchi SA, Thadhani R, Bhan I. Vitamin D-binding protein modifies the vitamin D-bone mineral density relationship. J Bone Miner Res 2011; 26:1609-1616

38. Bhan I, Powe CE, Berg AH, Ankers E, Wenger JB, Karumanchi SA, Thadhani RI. Bioavailable vitamin D is more tightly linked to mineral metabolism than total vitamin D in incident hemodialysis patients. Kidney Int 2012; 82:84-89

39. Jemielita TO, Leonard MB, Baker J, Sayed S, Zemel BS, Shults J, Herskovitz R, Denburg MR. Association of 25-hydroxyvitamin D with areal and volumetric measures of bone mineral density and parathyroid hormone: impact of vitamin D-binding protein and its assays. Osteoporos Int 2015;

40. Aloia J, Mikhail M, Dhaliwal R, Shieh A, Usera G, Stolberg A, Ragolia L, Islam S. Free 25(OH)D and the Vitamin D Paradox in African Americans. The Journal of clinical endocrinology and metabolism 2015; 100:3356-3363

41. Schwartz JB, Lai J, Lizaola B, Kane L, Markova S, Weyland P, Terrault NA, Stotland N, Bikle D. A comparison of measured and calculated free 25(OH) vitamin D levels in clinical populations. The Journal of clinical endocrinology and metabolism 2014; 99:1631-1637

42. Jeffery LE, Wood AM, Qureshi OS, Hou TZ, Gardner D, Briggs Z, Kaur S, Raza K, Sansom DM. Availability of 25-Hydroxyvitamin D3 to APCs Controls the Balance between Regulatory and Inflammatory T Cell Responses. Journal of immunology 2012; 189:5155-5164

Click here to download Table Table 1.docx

Table 1. Effect of vitamin D2 or vitamin D3 on indexes of bone structure and turnover in mice

Age/sex/diet	N	BV/TV (%)	Tb.N (*n*/mm^2)	Tb.Sp (μm)	Tb.Th (μm)	ES/BS (%)	MS/BS (%)	OS/BS (%)	MAR (μm/d)	BFR/BS (μm^3/μm^2/d)
Mice 8 weeks										
Female										
D2	7	8.45 ± 0.61	2.99 ± 0.13	309.72 ± 15.23	28.11 ± 1.16	4.48 ± 0.36	4.99 ± 0.82	6.57 ± 1.70	1.41 ± 0.06	25.14 ± 3.72
D3	7	7.13 ± 1.10	2.64 ± 0.24	373.73 ± 39.67	26.26 ± 1.51	1.73 ± 0.37	4.45 ± 0.54	6.78 ± 0.91	1.07 ± 0.13	18.24 ± 4.42
Male										
D2	8	12.63 ± 0.94	3.93 ± 0.22	228.13 ± 15.23	31.99 ± 1.11	3.65 ± 0.72	9.64 ± 1.69	3.08 ± 0.83	1.07 ± 0.03	37.75 ± 6.91
D3	8	12.58 ± 0.77	4.03 ± 0.10	218.31 ± 7.28	31.23 ± 1.67	2.29 ± 0.35	9.17 ± 0.84	3.37 ± 0.74	0.99 ± 0.05	33.53 ± 3.91
2-way ANOVA *p* value										
Sex		**< 0.0001**	**< 0.0001**	**< 0.0001**	**0.0038**	0.7866	**0.0003**	**0.0036**	**0.0105**	**0.0103**
Diet		0.4439	0.4816	0.2238	0.3589	**0.0003**	0.6516	0.8171	**0.0107**	0.2804
Sex x diet		0.4756	0.2212	0.1014	0.6983	0.1713	0.9761	0.9714	0.0945	0.7929
Mice 16 weeks										
Female										
D2	6	6.15 ± 0.29	2.14 ± 0.12	445.79 ± 25.28	28.84 ± 0.77	2.24 ± 0.53	8.84 ± 1.59	2.9 ± 0.50	1.01 ± 0.08	31.73 ± 5.39
D3	8	5.05 ± 0.43	1.76 ± 0.12	559.88 ± 47.52	28.67 ± 0.98	2.28 ± 0.32	9.23 ± 1.11	4.23 ± 1.45	0.89 ± 0.06	30.28 ± 3.95
Male										
D2	8	14.48 ± 1.50	4.00 ± 0.23	220.62 ± 17.37	35.60 ± 2.01	0.75 ± 0.31	4.01 ± 0.82	0.77 ± 0.31	0.65 ± 0.05	9.80 ± 2.59
D3	6	11.05 ± 0.84	3.46 ± 0.18	261.28 ± 15.33	31.82 ± 1.23	1.25 ± 0.14	2.77 ± 0.32	0.53 ± 0.30	0.64 ± 0.04	6.27 ± 0.47
2-way ANOVA *p* value										
Sex		**< 0.0001**	**< 0.0001**	**< 0.0001**	0.0026	**0.0051**	**< 0.0001**	**0.0236**	**< 0.0001**	**< 0.0001**
Diet		**0.0358**	**0.0170**	**0.0168**	0.1905	0.6971	0.6892	0.6733	0.4706	0.4881
Sex x diet		0.2624	0.6579	0.2338	0.2309	0.7868	0.4419	0.4005	0.2565	0.7711

Click here to download Table Table 2.docx

Table 2. Effect of vitamin D2 or vitamin D3 on osteoclast and osteoblast indexes in mice

Age/sex/diet	N	N.Oc/B.Pm (*n*/μm)	N.Oc (*n*)	N.Oc/T.Ar (*n*/mm²)	Oc.S/BS (%)	N.Ob/B.Pm (*n*/μm)	N.Ob (*n*)	N.Ob/T.Ar (*n*/mm²)	Ob.S/BS (%)
Young mice (8 weeks)									
Female									
D2	7	0.72 ± 0.09	8.71 ± 1.06	3.35 ± 0.43	1.84 ± 0.22	9.60 ± 1.65	117.57 ± 19.71	44.45 ± 7.09	13.41 ± 2.02
D3	7	0.21 ± 0.03	2.00 ± 0.31	0.80 ± 0.12	0.55 ± 0.10	4.09 ± 0.77	44.29 ± 9.84	18.00 ± 4.11	5.47 ± 1.08
Male									
D2	8	0.50 ± 0.08	9.75 ± 1.74	3.09 ± 0.54	1.58 ± 0.29	1.87 ± 0.29	35.37 ± 4.89	11.25 ± 1.56	2.82 ± 0.52
D3	8	0.35 ± 0.05	7.13 ± 0.95	2.20 ± 0.29	0.98 ± 0.12	2.72 ± 0.38	54.50 ± 6.54	16.87 ± 2.10	3.69 ± 0.64
2-way ANOVA *p* value									
Sex		0.6134	**0.0151**	**< 0.0001**	0.6908	**< 0.0001**	**0.0033**	**0.0003**	**< 0.0001**
Diet		**< 0.0001**	**0.0005**	**< 0.0001**	**< 0.0001**	**0.0139**	**0.0223**	**0.0165**	**0.0051**
Sex x diet		**0.0133**	0.0959	**0.0010**	0.1043	**0.0013**	**0.0003**	**0.0005**	**0.0007**
Mature mice (16 weeks)									
Female									
D2	6	0.44 ± 0.11	4.17 ± 0.95	1.42 ± 0.32	1.22 ± 0.31	4.28 ± 0.47	42.33 ± 5.62	14.49 ± 2.08	6.51 ± 0.59
D3	8	0.38 ± 0.07	3.00 ± 0.58	1.04 ± 0.20	0.97 ± 0.25	3.95 ± 0.57	30.17 ± 4.03	10.71 ± 1.54	5.45 ± 0.88
Male									
D2	8	0.14 ± 0.05	3.50 ± 1.23	1.11 ± 0.38	0.54 ± 0.19	0.48 ± 0.11	9.63 ± 2.15	3.11 ± 0.70	0.77 ± 0.17
D3	6	0.20 ± 0.03	3.33 ± 0.49	1.07 ± 0.14	0.63 ± 0.08	0.86 ± 0.25	14.00 ± 3.88	4.67 ± 1.37	1.25 ± 0.45
2-way ANOVA *p* value									
Sex		**0.0024**	0.8458	0.6115	**0.0346**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**
Diet		0.9852	0.4398	0.4598	0.7340	0.9816	0.2891	0.3717	0.5667
Sex x diet		0.4052	0.5611	0.5406	0.4462	0.4393	0.0789	0.1075	0.2408

Figure 1

Click here to download Figure Figure 1.tif

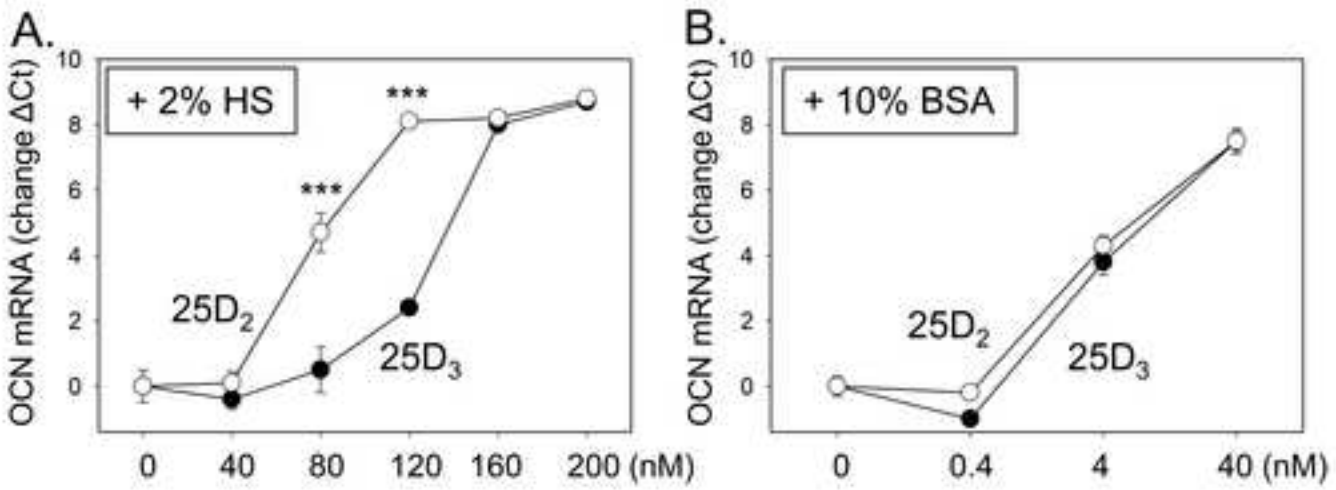


Figure 2

Click here to download Figure Figure 2.tif

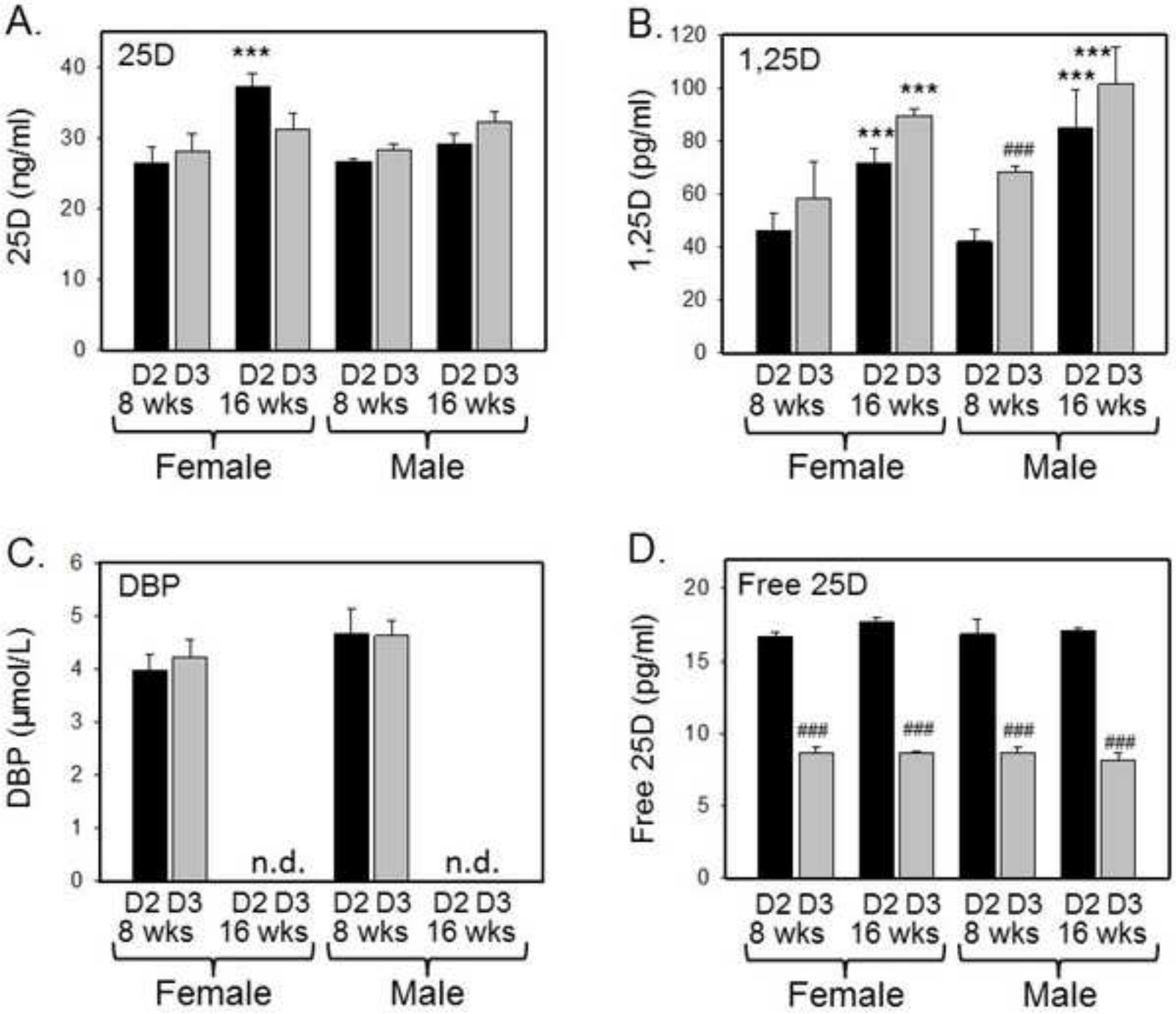


Figure 3

Click here to download Figure Figure 3.tif

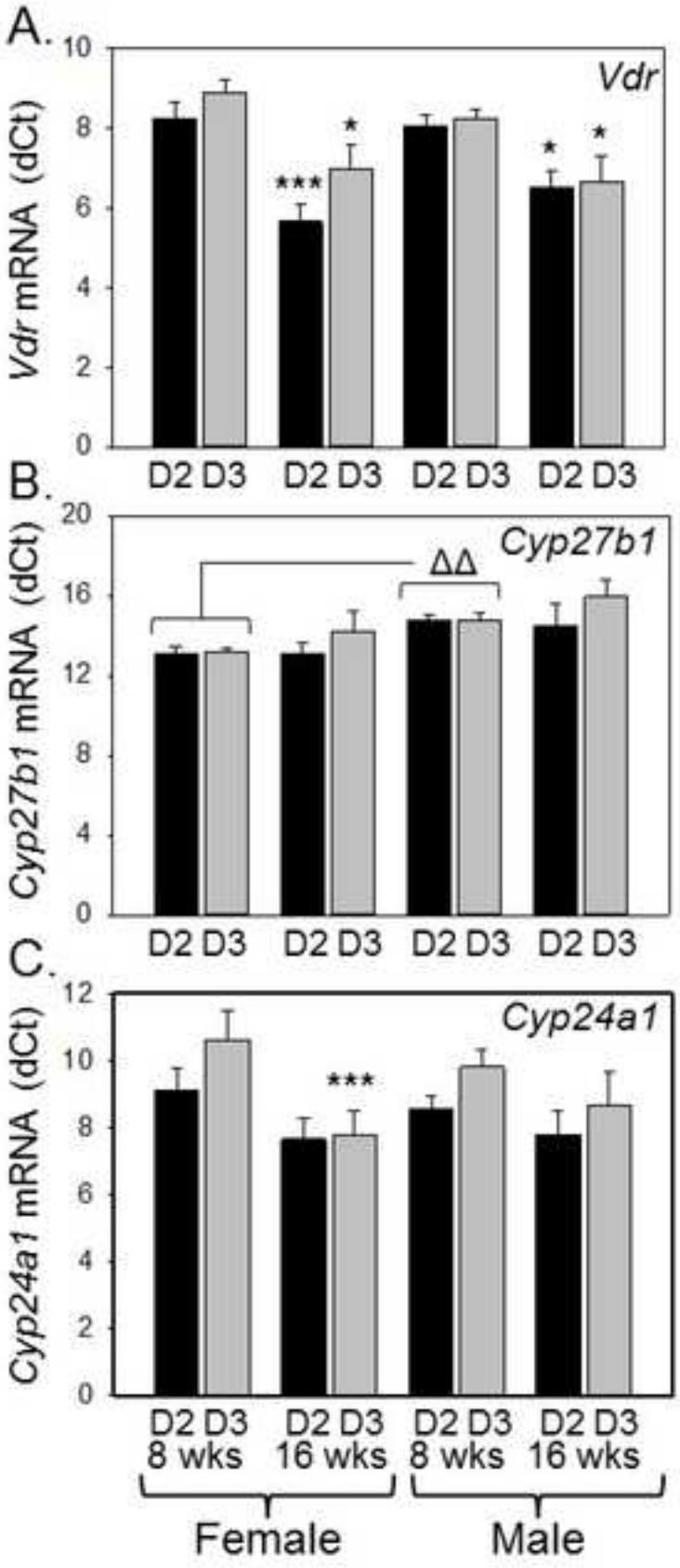


Figure 4

Click here to download Figure Figure 4.tif

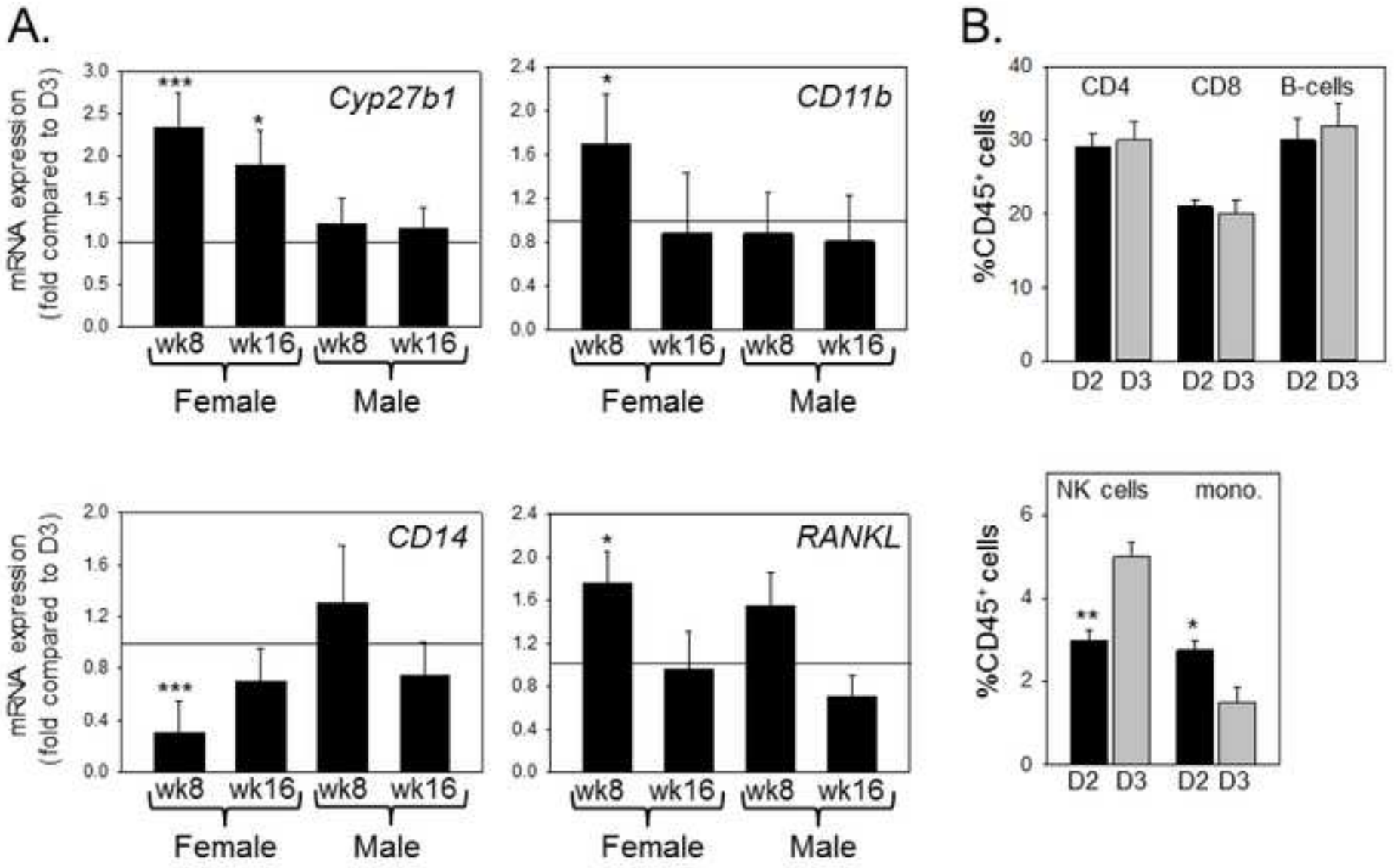


Figure 5

Click here to download Figure Figure 5.tif

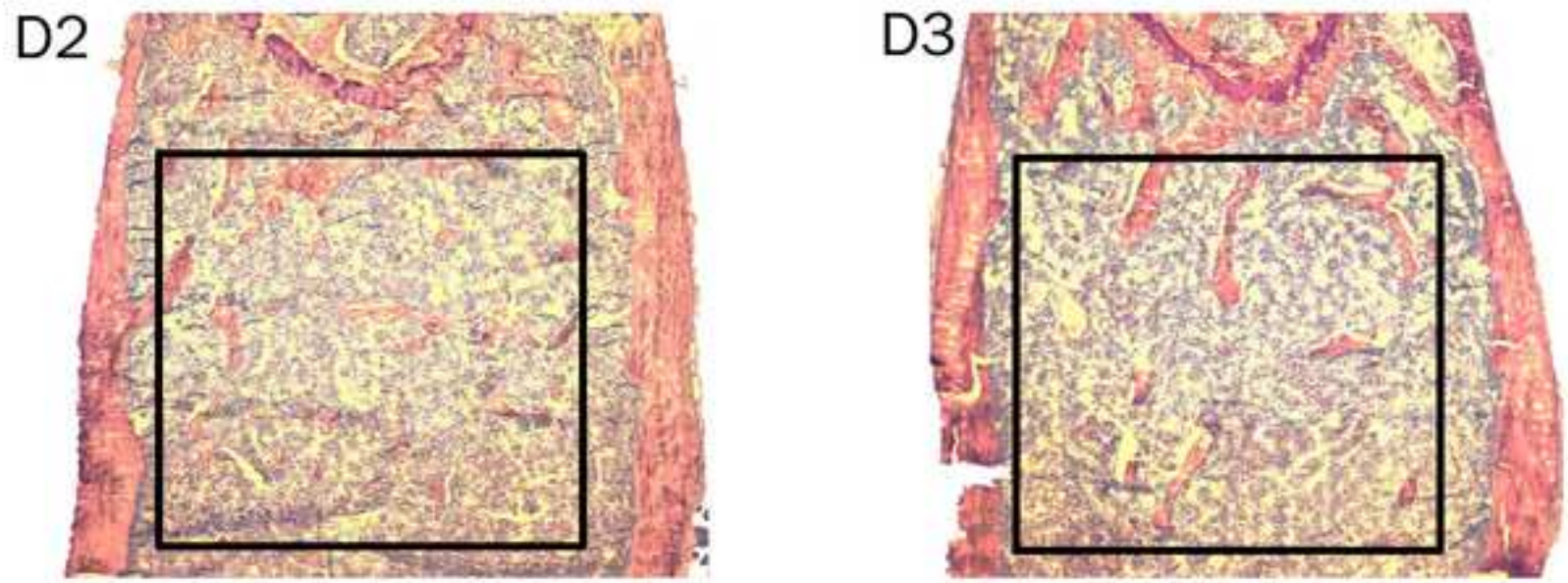


Figure 6

Click here to download Figure Figure 6 revised.tif

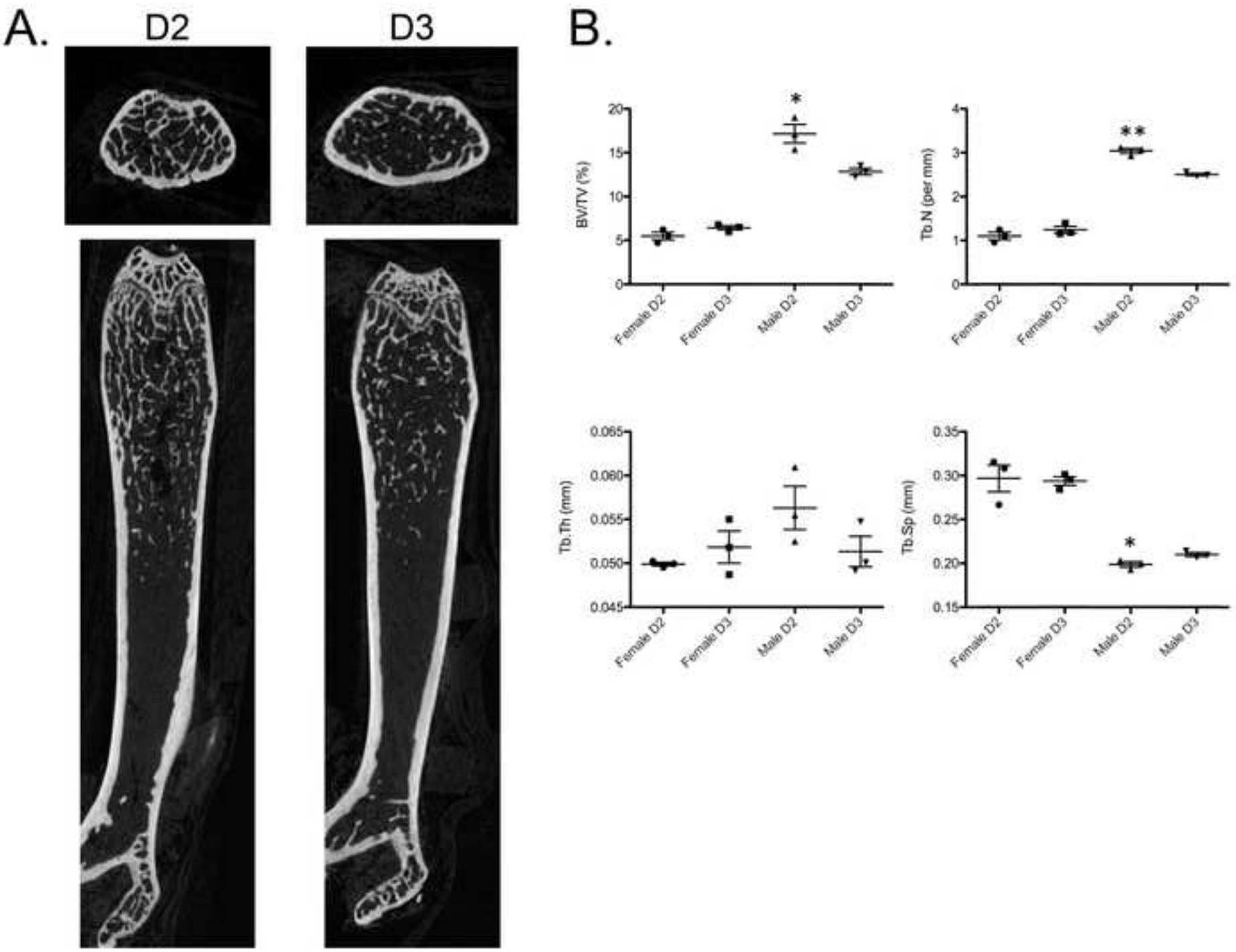
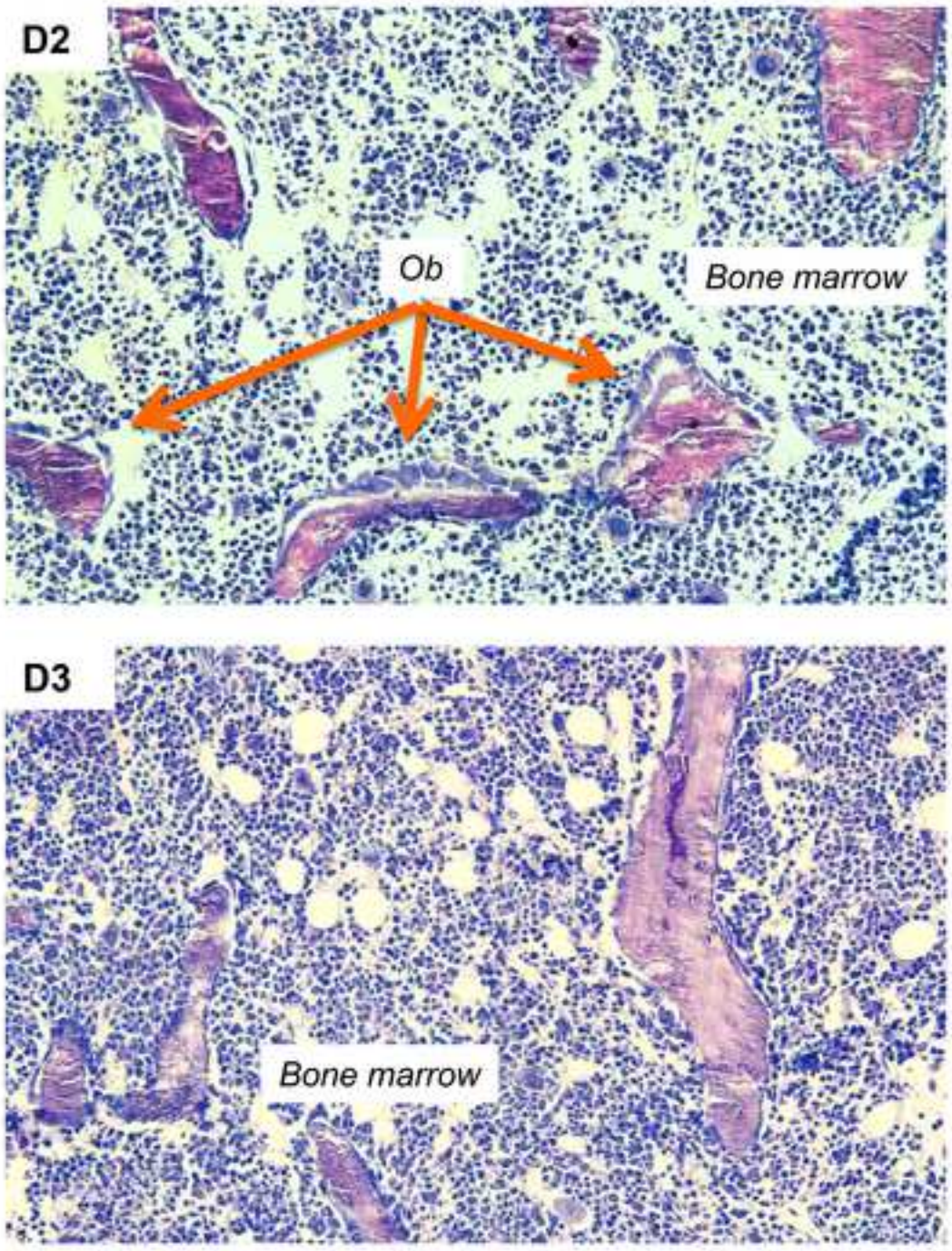


Figure 7

Click here to download Figure Figure 7.tif



Antibody Table

Peptide/protein target	Antigen sequence (if known)	Name of Antibody	Manufacturer, catalog #, and/or name of individual providing the antibody	Species raised in; monoclonal or polyclonal	Dilution used
CD45		30-F11	BD Biosciences 550994	Rat monoclonal	1/100
CD4		RM4-5	BD Biosciences 553048	Rat monoclonal	1/100
CD8a		53-6.7	BD Biosciences 553035	Rat monoclonal	1/100
B220		RA3-6B2	BD Biosciences 558108	Rat monoclonal	1/100
NK1.1		PK136	BD Biosciences 553164	Mouse monoclonal	1/250
CD11b		M1/70	BD Biosciences 553311	Rat monoclonal	1/100
CD3		17A2	BD Biosciences 555274	Rat monoclonal	1/250
F4/80		BM8	eBioscience 11-4801-85	Rat monoclonal	1/100